SYNOPSIS

on

Study of genetic diversity of *Aconitum heterophyllum* and its root microbiome

By

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1. Background

The genus *Aconitum*, commonly known as Aconite, belongs to family Ranunculaceae and is one of the most species-rich genera in India. *Aconitum heterophyllum* Wall. ex Royle, is a perennial medicinal herb of North-Western Himalayas (Dhar and Kachroo, 1983). *A. heterophyllum* is endemic to the Himalayan region at an altitude of 2400-4500 m (Bhatt et al. 2014; Jabeen et al. 2013). It commonly known as Atis in Hindi, Aruna of visa in Sanskrit and Atees in Urdu. *A. heterophyllum* is known for the medicinal value of its roots referred to as Ativisa. The roots of the plant are reported to exhibit a wide range of pharmacological activities but is mainly known for its Anti-pyretic (Verma *et al.*, 2010) and Anti-diarrheal (Singh and Chaturvedi, 1982) potential. They are rich source of diterpene alkaloids and are known to contain atisine, a low-toxic alkaloid belonging to the category of simpler aconite alkaloids. In ancient texts, it has been recommended for treating obesity, diarrhea piles, etc.

A. heterophyllum is categorized under the endangered category of International Union for the Conservation of Natural Resources (IUCN) of the Red Data Book (Ved et al., 2003). Rapid depletion of the species can be attributed to its low population density in nature (Dhar and Kachroo, 1983) along with overexploitation due to unsustainable collection due to high market value (Nautiyal et al., 2002) of the tuberous roots. Its sustainable use and conservation of the natural populations and sustainable utilization is the need of the hour. For this, it is imperative to study the current population status of the selected species. Genetic diversity plays an important role in adaptation of the species in nature and accounts for the possibility of a population to evolve and adapt to changes in environment. However, Limited studies have been carried out to assess the genetic diversity of the genus *Aconitum* in India. Furthermore, understanding the rhizosphere of medicinal, endemic plant species will be vital for bioprospection of ecto-and endophytic microbes and in selecting suitable sites for *ex-situ* conservation and cultivation. Effective agricultural practices could also be developed to improve root biomass of existing variety by studies on soil-microbe-plant interactions as specific microbe interactions are known to be associated to endemism of species.

2. Objectives

Broad objectives of the present research are summarized below:

2.1. Characterization of microsatellite markers in A. heterophyllum.

2.1.1. Utilization of PCR based molecular markers (SSRs derived from ISSR-SSR amplicons) for generating microsatellites and by exploring cross-species transferability- an approach for generation of microsatellite markers for related species.

2.2. Assessment of genetic diversity in population of A. heterophyllum.

2.2.1. Phenotypic assessment of accessions of A. heterophyllum from Uttarakhand and Himachal Pradesh states of India.

2.2.2. Species authentication and analysis of genetic diversity using characterized markers.

2.3. Study of the Root microbiome using metagenomic approach.

2.3.1. Assessment of colonization pattern of the root microbiome in endemic A. heterophyllum and by targeted gene sequencing.

2.3.2. Identification of unique microbes using targeted gene sequencing, by comparison of wild and cultivated root microbiome of A. heterophyllum.

3. Material and Methods

3.1 Identification of habitats and collection of samples and collection of samples

Habitat identification of the targeted species- *Aconitum heterophyllum* was done by exploring literature and local leads from Uttarakhand and Himachal Pradesh states of India. Botanical Identification was done and documented through Herbarium preparation. The Geographical coordinates were mapped using the geographical coordinates. Leaf samples were collected for genetic diversity studies while root tip and base parts were collected form the identified populations representing the wild and cultivated population. Excess soil was removed from the roots by shaking, leaving roots and firmly adhering soil, the rhizosphere soil.

3.2. DNA extraction and Selection of primers for assessment of genetic diversity

DNA was isolated from the collected accessions using Qiagen DNeasy plant DNA isolation mini kit (Qiagen, USA) and stored at -20°C. To detect polymorphism among the collected accessions, microsatellite loci were identified using the following approaches:

3.2.1. Transferability of SSR markers- Flanking sequences to microsatellites are reported to exhibit slower mutation rate than SSR region, permitting their sequence conservation across species or genera (Holmen *et al.*, 2009). This homology allows the amplification of primers designed for one species to other members of the same species or genera (cross-species/cross-genera amplification or transferability). To identify microsatellites in *A. heterophyllum*, a total of 44 pairs of primers were selected based on reported number of alleles in species of genus *Aconitum*. Transferability was assessed using these pair of primers in *A. heterophyllum*.

3.2.2. Anchored microsatellite-primed PCR (AMP-PCR)- Di- or trinucleotide repeats with 5'- or 3'- anchors serves as single PCR primer for amplification of microsatellite regions with the repeat (Zietkiewicz *et al.*, 1994). 5'- anchored primers designed by Fisher *et al.*, (1996) were used in the present study as functional 5'-anchors ensure that the targeted microsatellite is part of the product. The degenerate primer (PCT4) designed with the sequence KKVRVRV$(CT)_6$ (where K = G/T, V = G/C/A and R = G/A) was tested in *A. heterophyllum*. Six degenerate 5'-anchored primers with different repeat motifs (Caldeira *et al.*, 2002) [KKVRVRV$(CT)_6$, KKVRVRV$(CT)_{10}$, KKVRVRV$(GA)_{10}$, KKVRVRV$(CA)_6$, KKVRVRV$(AGA)_6$ and KKVRVRV$(GT)_6$] were used for amplification of microsatellite region of the targeted species of *Aconitum*. DNA sequence analysis of the amplified fragments was done using Oxford Nanopore MinION sequencer (ONT, Oxford Nanopore Technologies, UK) to confirm the amplification of microsatellite region. Perl scripts with default parameters from MISA (MIcroSAtellite) identification tool (Beier *et al.*, 2017) were used to verify targeted SSR repeat.

3.2.3. Data analysis

Population genetic and analysis of molecular variance (AMOVA) was executed using the software package GenAIEx 6.5 (Peakall and Smouse, 2012). Population structure was studied using the software package STRUCTURE 2.3.4 (Pritchard *et al.*, 2000) and STRUCTURE HARVESTER Program (Earl and Von Holdt, 2012). For cluster analysis, the computer package NTSYSpc 2.2 (Rohlf, 1990) was used.

3.3. Root microbiome studies

3.3.1 DNA isolation of root-associated microbiota

The protocol briefed by Edwards et al. (2015) using washing and sonicating steps to separate the rhizosphere fraction of the root-associated microbiota was used. DNA was isolated from the soil suspension using modified procedure of Qiagen DNeasy PowerSoil kit (Qiagen, USA).

3.2.2. The Identification of Root-Associated Microbiomes

In the present study the marker genes, 16S rRNA was used to study the root-associated bacterial microbiome in wild and cultivated populations of *A. heterophyllum*. In the process of identification, the 16S rRNA gene is initially amplified through polymerase chain reaction (PCR) using primers designed to bind to these conserved regions, followed by subsequent sequencing. The forward (16S_27F: 5'- AGAGTTTGATCATGGCTCAG -3') and reverse (16S_1492R: 5'- CGGTTACCTTGTTACGACTT -3') set of primers were used for amplification of the complete 16 rRNA gene.

3.2.3. Sequencing of amplicons and Taxonomic analysis

Sequencing of the amplified fragments was done using Oxford Nanopore MinION sequencer (ONT, Oxford Nanopore Technologies, UK), Amplicons were quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, US), and 1 µg amplicon was used for library preparation. The raw reads (fastq) were processed to remove adapter sequences and low-quality bases using Porechop. The trimmed reads were processed to Kraken2 tool (Wood et al., 2019), which classifies the reads using k-mer method against GREENGENE and SILVA databases. Abundance tables were generated using pavian and processed for further downstream analysis. Alpha diversity was derived using indices of which Chao1 and ACE represent the richness of the sample and Shannon, Simpson, InvSimpson and Fisher represent both richness and relative abundance. To identify statistically significant difference among OTUs abundance between the samples, Fisher's exact test was carried out using STAMP, a graphical software package that provides statistical hypothesis tests and exploratory plots (Parks *et al.,* 2014).

4. Result and Discussion

4.1. Identification of habitats and collection of samples

High altitude regions (2500-4800 m) of Uttarakhand and Himachal Pradesh were identified as habitats of A. *heterophyllum* as reported in the literature. Samples were collected from these habitats between August 2020 and October 2021. Samples were collected from eight populations and the population sites were mapped using the geographical coordinates in geographic information system program QGIS. Sampling was done randomly from each population based on availability of individuals, as population was found to be highly fragmented and small, witnessing extremely low population density of the species. Voucher specimens were deposited in the herbarium of Taxonomy department of the University of Trans Disciplinary Health Sciences and Technology, Bangalore. Thirty-six leaf samples were collected for characterization of microsatellite markers and assessment of genetic diversity. For rhizospheric studies a total of 10 plant samples were collected. Out of this, 5 samples represented the wild population and the remaining 5 represented the cultivated population.

4.2. Characterization of microsatellite markers

4.2.1. Through transferability of SSR markers- Based on amplification and polymorphism observed in the targeted species, 10 primers were selected. The present study reports successful cross-species transferability of microsatellite markers from different species of the genus *Aconitum* to *A. heterophyllum* species.

4.2.2. Through Anchored microsatellite-primed PCR (AMP-PCR)- 5'-anchored primers with the targeted repeat motifs [KKVRVRV$(CT)_6$, KKVRVRV$(CT)_{10}$, KKVRVRV$(GA)_{10}$, KKVRVRV$(CA)_6$, KKVRVRV$(AGA)_6$ and KKVRVRV$(GT)_6$] successfully amplified the microsatellite region in *A. heterophyllum*. This was confirmed by the DNA sequence analysis of the amplified fragments which showed the presence of SSR sequences. Out of six 5'-anchored primers tested, four were selected based on the polymorphism observed in *A. heterophyllum*. DNA sequencing of the amplified fragments using Oxford Nanopore MinION sequencer generated 3.6 M reads with 2.98 Gb estimated bases. Identification of microsatellites using MISA identification tool confirmed the presence of targeted SSR repeats in the amplicons.

4.3. Genetic diversity and Population Structure

Four AMP-PCR primers targeting di- and trinucleotide repeats along with ten microsatellite markers identified by accessing transferability, were used to study the genetic diversity and population structure in *A. heterophyllum*. Highest genetic diversity was observed in AHBC (Bedni-Chamoli, Uttarakhand) population with Shannon's information index (I) of 0.290 and unbiased expected heterozygosity (uH_e) of 0.221. The lowest genetic diversity was observed in AHS population with I=0.207 and uH_e=0.159. The results from AMOVA indicated higher variation among the populations (92%) and very low variation of 8% within the population with total estimated variance (EV) of 1.886. Further, observed genetic differentiation coefficient (F_{st}) and gene flow (N_m) at species level was 0.490 and 0.932, respectively. The population structure analysis of 36 accessions of *A. heterophyllum* from eight distant geographical locations estimated optimum subpopulation of K=2. The accessions were grouped in two subpopulations and no admixtures were reported. Cluster analysis using UPGMA by estimating Dice coefficient of similarity reported two major clusters. Overall, the results indicated low genetic diversity, both at population (uH_e=0.159- 0.224) and species level (uH_e=0.194).

4.4. Root microbiome studies

PCR amplification of the 16S rRNA gene from the genomic DNA of samples was conducted using a pair of primer that targets full length of 16S gene. Amplification was checked by Agarose gel electrophoresis and amplicons of approx. 1500 bp observed. DNA sequencing of the amplified fragments using Oxford Nanopore MinION sequencer generated 5.72 M reads with 5.12 Gb estimated bases. Q7 filtering using MinIONQC recovered 4.18 Gb bases with mean quality (Q) score of 10.1. An average of 99.76% reads were classified out of which 100% were bacterial reads. While no viral, fungal, or protozoan reads were reported, 0.24% reads were unclassified.

4.4.1. Detection of Operational Taxonomic Units (OTUs)

The OTUs were detected in the samples using Greengenes and Silva reference databases. Phylum abundance using Greengenes and Silva reference databases reported similar phylum in the samples. Major phylum reported by the reference databases are Proteobacteria,

Cyanobacteria, Acidobacteria, Planctomycetes, Bacteroidetes, Actinobacteria, etc. Further, all the samples were observed to contain similar phylum.Genus abundance using Greengenes and Silva reference databases reported similar genus in the samples. Similar genus was observed in all wild and cultivated accessions. Some of the major genus observed using the reference databases in the samples were Agrobacterium (Rhizobium in Silva), Bacillus, Bradyrhizobium, Flavisolibacter, Gemmata and Massilia (Unclassified under family Oxalobacteraceae in Greengenes). Species abundance was reported by referring Greengenes database. A total of 1981 OTUs were identified.

4.4.2. Alpha and Beta diversity

Richness (number of taxonomic groups) and evenness (distribution of abundances of the groups) was depicted using different alpha diversity metrices. Indices Chao1 and ACE represented richness of the samples. Compared to the wild sample from Chamoli-Uttarakhand (1A-BC-W), the cultivated sample (1A-GC-C) represented lower richness as low Chao1 and ACE coefficient was reported in this sample. However, the cultivated samples from Dayara-Uttarkashi (2B-DU-C), Khaliya-Munsyari (3B-KM-C) and Chandrashila-Uttarakhand (4B-TCU-C) reported higher species richness in terms of Chao1 and ACE as compared to their wild counterparts 2A-DU-W, 3A-KM-W and 4A-TCU-W respectively. The Beta Diversity was derived using PCoA (Principal Coordinate Analysis). The wild and cultivated samples Chamoli-Uttarakhand (1A-BC-W and 1B-GC-W) were clustered together in the plot. The second cluster included wild and cultivated accessions from Khaliya-Munsyari and Tindi-Lahaul Spiti. The third cluster contained wild and cultivated accessions from Dayara-Uttarkashi and Chandrashila-Uttarakhand Overall, three clusters were observed, but it may be noted that wild and cultivated counterparts of respective diverse populations were clustered together, thus representing low wild versus cultivated diversity.

4.4.3. Unique OTUs in wild versus the cultivated populations

Significant number of unique OTUs were reported in the wild samples when compared to the cultivated counterparts. Further, it was found through literature that many of these species were reported to have significant roles in plant growth and metabolism. Fisher's exact test statistically reported significant difference among OTUs abundance between the wild and

cultivated samples. A total of 316 unique OTUs were observed in the wild sample (1A-BC-W) versus the cultivated sample (1B-GC-C) of Chamoli-Uttarakhand. A total of 195 unique OTUs were reported by Fisher's exact test in the wild sample (2A-DU-W) versus the cultivated sample (2B-DU-C) of Dayara-Uttarkashi-Uttarakhand. Further, a total of 211 unique OTUs were reported by Fisher's exact test in the wild sample (3A-KM-W) versus the cultivated sample (3B-KM-C) of Khaliya-Munsyari-Uttarakhand and a total of 163 unique OTUs were reported by Fisher's exact test in the wild sample (4A-TCU-W) versus the cultivated sample (4B-TCU-C) of Chandrashila-Uttarakhand. Another 270 unique OTUs were reported by Fisher's exact test in the wild sample (5A-LHP-W) versus the cultivated sample (5B-LHP-C) of Tindi-Lahaul Spiti-Himachal.

5. Conclusion

A. heterophyllum is a valuable medicinal plant known for its medicinal properties, particularly its dried roots, which are widely used in traditional medicines. The overexploitation and unsustainable collection practices are causing a rapid decline in the plant's population in the wild. Genetic diversity in *A. heterophyllum* was studied using characterized microsatellite markers. The microsatellites in the targeted species was characterized by exploring cross species transferability and by using Anchored microsatellite-primed PCR (AMP-PCR). The study reports successful cross-species transferability of microsatellite markers from different species of the genus *Aconitum* to *A. heterophyllum* species. Further, polymorphism in *A. heterophyllum* was successfully detected using AMP-PCR and markers were characterized. The present study found only a few isolated populations of *A. heterophyllum*, and most of them consisted of only a small number of individuals. The study also revealed that these populations have low genetic diversity, significant genetic differentiation, and limited gene flow among them. To protect this species, urgent measures are needed to preserve its natural habitats. One effective conservation strategy is to introduce genetic diversity into these populations through methods such as seed dispersal and artificial pollination. Conservation plots or designated areas should be established to safeguard populations with significant genetic variation, ensuring the protection of the natural habitats of A. heterophyllum. This holistic approach will contribute to the long-term survival and sustainability of this critically endangered medicinal plant.

Further, the rhizospheric root microbiome of the targets species was studied using 16S rRNA metagenomic approach. Unique bacteria were identified by comparison of wild and cultivated root microbiome of *A. heterophyllum*. Several rhizospheric bacteria with known beneficial effects on the plant, the Plant Growth Promoting Rhizobacteria (PGPR), associated with the wild populations are reported in the present study. The identified microbes can be utilized for promoting sustainable and efficient cultivation of the species, fostering a balanced and mutually beneficial relationship between plants and beneficial microorganisms in the soil. These PGPR can also stimulate the development of a well-branched and robust root system. Improved root architecture enhances nutrient and water absorption, leading to better overall plant performance and better yield of Ativisa- the medicinal important part of the targeted species.

6. Publications

1. "**Ethnomedicinal Properties and Conservation issues of an endangered Medicinal Plant-*Aconitum heterophyllum* Wall. ex Royle**". Published in Medicinal Plants - International Journal of Phytomedicines and Related Industries.
2. "**Genetic diversity and population structure of *Aconitum heterophyllum*- an endangered plant from North-western Himalayas using microsatellite markers**". Published in Medicinal Plants - International Journal of Phytomedicines and Related Industries.

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